

To: Dr. A. C. Lilly

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From: A. H. Warfield, R. D. Kinser and D. J. Ayers

Subject: TSNA Program Operational Plans for 1990.

**OBJECTIVE(S):** (1) To design a first generation laboratory model of a product by 1991 with MS TSNA (TSNA/mg TPM) delivery reduced 90% relative to the TPM-corrected TSNA delivery of a 1987 full-flavored, blended cigarette. (2) To design a second generation laboratory model of a product by 1993 with MS TSNA delivery (TSNA/mg TPM) delivery reduced 95% relative to the TPM-corrected TSNA delivery of a 1987 full-flavored, blended cigarette, utilizing technology based on a fundamental understanding of NA formation.

**STRATEGIES:**

**REDUCTION OF MS TSNA BY REDUCING ENDOGENOUS TSNA & PYROSYNTHETIC TSNA PRECURSORS IN FILLER**

1. Reduce MS TSNA by selective removal of TSNA, amine precursors, and/or nitrosating agent precursors from filler.
2. Reduce MS TSNA by biochemical alteration(s) to tobacco leading to removal of alkaloid precursors of TSNA.

**REDUCTION OF MS TSNA BY INHIBITING THE PYROSYNTHESIS OF TSNA**

3. Reduce the levels of pyrosynthesized MS TSNA by incorporation into the cigarette design those aspects of oriental filler which result in an absence of significant TSNA pyrosynthesis from oriental tobacco.
4. Reduce the levels of pyrosynthesized MS TSNA by decreasing the reactivity to nitrosation of the amine precursor(s), or blocking reaction pathways which form nitrosating agent(s) or which yield TSNA from the nitrosating agents.

**REDUCTION OF MS TSNA BY ENHANCING DECOMPOSITION OF TSNA**

5. Evaluate the enhancement of TSNA decomposition during smoking as a method for reducing TSNA delivery.

**REDUCTION OF MS TSNA BY ALTERING PHYSICAL/CHEMICAL PARAMETERS OF CIGARETTES**

6. Reduce the levels of pyrosynthesized MS TSNA by alterations in cigarette construction parameters.
7. Reduce the levels of pyrosynthesized MS TSNA by manipulation of filler salt content.
8. Reduce the levels of pyrosynthesized MS TSNA by manipulation of casings typically used in cigarettes but missing from the reference cigarette.

TACTICS AND TIMETABLE:

1990

REDUCTION OF MS TSNA BY REMOVAL OF ENDOGENOUS TSNA & TSNA PRECURSORS

Selective Removal From Cured Filler Strategy:

Complete scale-up of current 95% EtOH extraction process to  
yield material for laboratory model:

Reverse flow during extraction using large column.....	Feb. 15
Use smaller columns in series.....	March 1
Extract a blend instead of individual fillers.....	March 15
Investigate effect of extraction time.....	April 1
Plan aging study on solvent-extracted fillers.....	April 1

Investigate additional means of removing UN from filler:

Wash DBC Bu filler with dilute aqueous citric and mineral acid.....	March 9
Wash DBC Bu filler with LaCl <sub>3</sub> or other salts to remove Ca.....	March 31
Complete planning for additional modifications of fillers by aqueous washing.....	March 31

Complete aging study and prepare report with  
recommendations for storage..... June 30

Collaborate with ARD and PRD in development of SCFE  
methodology capable of removing minor alkaloids from filler.. ongoing

Biochemical Alterations to Tobacco Strategy:

Continue <sup>3</sup>H-SAM radiolabeling studies to label PMT:

Validate analysis methods.....	Feb. 9
Validate control experiments.....	Feb. 16
Label and analyze PMT samples.....	March 16
Evaluate company(s) offering 2D gel analysis to determine best replacement for Protein Databases Inc....	March 31
Send labeled samples for 2D gel analysis.....	March 31

Express overly expressed root clones in *E. coli* and assay  
for PMT activity from the *E. coli* cell extracts..... March 31

Provide PMT prep in amount and purity necessary for  
sequencing and send to outside vendor for sequencing..... June 30

Initiate tobacco cell culture studies..... July 1

Develop manual DNA sequencing methodologies in-house..... July 31

Have defined oligonucleotide probes of PMT to be  
synthesized..... Sept. 30

Develop 2D gel analysis capabilities in-house..... Sept. 30

Sequence overly expressed root clones..... Oct. 31

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Initiate screening of cDNA library from root with  
oligonucleotide probes to identify PMT..... Dec. 31

Compare DNA sequences obtained in-house with other reported  
sequences using computer programs..... Dec. 31

Continue protein purification methods development in house..... ongoing

Continue to obtain PMT samples processed from the ammonium  
sulfate stage through the phenyl-Sepharose and DEAE  
"affinity" mode..... ongoing

Continue to provide purified PMT preps, as needed..... ongoing

Purify selected tobacco enzymes, as needed..... ongoing

Continue appropriate tobacco biochemistry studies..... ongoing

Continue biochemical alterations studies..... ongoing

Continue experiments relevant to next alkaloid biogenesis  
pathway to be modified..... ongoing

Amine Precursor Strategy:

Evaluate the role of unextracted nicotine (UN)  
in TSNA pyrosynthesis by the following:

Enzyme digestions of Bu marc after water extraction initiate  
to separate Nic-Y and NN-Y from marc..... Feb. 15

Write SOP for the determination of different forms  
of nicotine in tobacco and tobacco extracts..... March 1

Isolate >4 kD Nic-X and <4 kD Nic-X by GPC from hot initiate  
water solubles (with 6912)..... March 15

Determine TSNA in filler and smoke of SCFE Br, Bu  
and Or and the BWs from extracted fillers..... April 30

Determine time course of Nic-Y and NN-Y development in  
green, dry, and cured Bu tobacco..... June 30

Apply Nic-X and Nic-Y to BWs and evaluate as  
precursors for MS NNK..... Dec. 31

Identification of Nic-X and Nic-Y..... Dec. 31

Exploratory <sup>13</sup>C alkaloid tracer studies for  
unextracted precursors..... Dec. 31

Investigate appropriate fillers for unextracted alkaloids. ongoing

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Investigate proximate NNK precursor:

Develop plan for studying effect of nicotine oxidation products on TSNA pyrosynthesis in MS/SS smoke..... Feb. 15  
Develop derivatization/HPLC method for pseudooxynicotine and other secondary amines..... Mar. 15  
Add nicotone to filler and evaluate its effect on NNK formation..... March 31  
Apply derivatization method to filler and smoke..... May 30  
Develop plan to specifically address the reduction of NNK in tobacco..... June 30

REDUCTION OF MS TSNA BY INHIBITING PYROSYNTHESIS

Oriental Inhibitor Strategy:

Complete evaluation of eight Or varieties:

Data analysis using expert system software..... June 30  
Document study (including recommendations)..... Aug. 31

Extraction of Or filler to isolate potential inhibitor:

Test SCF extract for inhibitory activity..... Feb. 15  
Test fractions for inhibitory activity..... March 15  
If active fractions present, identify active components..... May 7  
Add Or inhibitor + antioxidant to low TSNA filler..... Aug. 31  
Smoke above to determine effect on MS TSNA..... Sept. 30

Plan study to develop a BCR-reconstituted material (with reduced TSNA delivery).....

March 31

Complete study and have the modified BCR-reconstituted material made in Pilot Plant.....

Aug. 31

Nitrosating Agent Strategy:

Initiate studies of role of NO and nicotine in combined gas stream to test for gas phase reaction & mechanism of NNK release....

March 31

Determine effect of diene release compounds on TSNA delivery:

Complete MS evaluation of diene release compound as NO scavenger.....

April 30

Carry out selected reactions of sugars with ammonia and/or amino acids in order to evaluate reaction products as scavengers of nitrosating agents during smoking:

Test various sugar-derived materials as nitrosating agent scavengers/pyrosynthesis inhibitors

-Complete MS evaluation of proline-Amadori compd..... May 31

-Reevaluate & determine future direction of approach. June 30

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Develop methodology to test flash heating of TPM and filler to determine if TSNA precursors for pyrosynthetic type reactions are present in TPM and filler and to define time/temperature window for reaction..... June 30

Test flash heating of TPM and filler to determine if TSNA precursors for pyrosynthetic type reactions are present in TPM and filler and to define time/temperature window for reaction..... Sept. 30

Finalize results from study on NO and NIC in nitrosation and document..... Sept. 30

Continue studies of nitrosation agent scavengers (evaluate reaction products as scavengers of nitrosating agents during smoking)..... ongoing

#### ALTERATION OF PHYSICAL/CHEMICAL PARAMETERS

Evaluate effect of paper porosity and packing density:  
 Carry out remainder of study..... Oct. 31  
 Use data obtained to design best cigarette construction for use in blended low TSNA product..... Dec. 31

#### PREPARATION OF FIRST GENERATION LOW-TSNA LABORATORY MODEL

Add ASC, sugars to blend of Or with extracted Bu & Br and evaluate effect on MS TSNA..... Jan. 15

Add OrCEL to blend of Or with extracted Bu & Br and evaluate effect on MS TSNA..... Feb. 28

Incorporate Low TSNA BCR reconstituted material into the above blend and evaluate..... Nov. 30

Determine MS TSNA on experimental filler modified as above..... Dec. 15

Request S/M assay on smoke from these cigarettes..... Dec. 31

Submit above for small scale subjectives..... Dec. 31

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ANALYTICAL METHODS DEVELOPMENT:

Evaluate the currently used SS chamber smoke collection method  
for artifactual NA formation and develop alternate smoke      initiate  
collection system if necessary..... March 31

Develop HPLC method (UV Vacancy Chromatog.) for  
nitrate/nitrite with lower limit of detection than      as time  
currently available at PM R&D..... permits

Develop SPE method for smoke TSNA workup..... as time  
permits

SUPPORT OF OTHER PROGRAMS:

Design and carry out study of effects of cigarette paper on  
nitrosamines in SS chamber..... May 31

Project ART support..... ongoing

Support of other PM facilities..... ongoing

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1991

Selective Removal From Cured Filler Strategy:

Optimize first generation low-TSNA model for TSNA reduction.

Attempt to incorporate design parameters determined by CSBW Program.

Formulate plans for refined first generation low-TSNA lab model based on use of a SCFE process designed to remove nicotine, endogenous TSNA, and alkaloid precursors of TSNA.

Biochemical Alterations to Tobacco Strategy:

Prepare anti-sense DNA constructs.

Prepare vector(s) for insertion of anti-sense constructs.

Transform tobacco leaf tissue, cells and protoplasts.

Regenerate transformed plantlets; select individuals with lowest nicotine.

Manipulation of Casings Strategy:

Plan and implement study to determine effect of casings typically used in cigarettes but missing from reference cigarettes.

Support of Other Programs:

Project ART Support.

Support of other PM facilities.

1992-1993

Amine Precursor, Nitrosating Agent, and Oriental Inhibitor Strategies:

Incorporate pyrosynthesis inhibitors, NO scavengers, NNK pyrosynthesis inhibitors (if available) into the first generation low-TSNA model to yield a second generation low-TSNA model.

Biochemical Alterations to Tobacco Strategy:

Further develop and test transformed tobacco plants.

Support of Other Programs:

Support of PM facilities.

1994

Transfer technology for low TSNA model.

Support of Other Programs:

Support of PM facilities

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RESOURCE ALLOCATIONS FOR 1990:

How are the personnel assigned to this program allocated?  
(These allocations are for personnel in the BCR Division)

	Professionals	Technicians
Extraction of Endogenous TSNA and TSNA Precursor Strategy:	1.20	0.50
Biochemical Alteration of Tobacco Strategy:	9.25	1.00
Oriental Inhibitor Strategy:	1.30	0.30
Amine Precursor Strategy:	2.40	0.50
Nitrosating Agent Strategy:	0.90	0.50
Cigarette Construction Parameters Strategy:	0.30	0.20
TOTAL	15.35	3.00

TRANSFER OF TECHNOLOGY:

The target date for the first generation laboratory model of a reduced TSNA product is 1991. An interim technology based primarily on lab-scale solvent extraction may have to be utilized until SCFE methodology capable of removing minor alkaloids (now being investigated) can be developed. When the latter has been accomplished, the corresponding technology will be transferred to Development. At that time, companion technologies involved in preparation of the low-TSNA filler can also be transferred to Development if deemed desirable.

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